Elevated Extracellular Calcium Concentrations Induce Type X Collagen Synthesis in Chondrocyte Cultures

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Abstract. Chondrocytes at different stages of cellular differentiation were isolated from the tarsal element (immature chondrocytes) and zones 2 and 3 (mature chondrocytes) of 12-d chick embryo tibiotarsus. The chondrocytes from the two sources differed in their cell morphologies, growth rate and production of type X collagen. In 24 h, zone 2 and 3 chondrocytes synthesized 800 times more type X collagen than tarsal chondrocytes. The effect of exogenous $CaCl_2$ (5 and 10 mM) on the synthesis of type X collagen by both mature and immature chondrocytes was tested. After a 72-h incubation of zone 2 and 3 chondrocytes with $CaCl_2$ type X collagen increased 8-fold with 5 mM and 10-fold with 10 mM Ca^{2+}. [^{3}H]Proline incorporation into culture medium and matrix macromolecules increased 11 and 32% with 5 and 10 mM $CaCl_2$, respectively. Type II collagen synthesis was not affected by elevated extracellular Ca^{2+} during this 72-h period.

Similar studies with tarsal chondrocytes demonstrated a time- and dose-dependent response to $CaCl_2$ with type X collagen levels reaching a 4-fold and 15-fold increase over controls with 5 and 10 mM Ca^{2+}, respectively, at 48 h. Elevated extracellular Ca^{2+} had no effect on cell proliferation. These observations offer the first direct evidence of the induction of type X collagen synthesis with elevated extracellular Ca^{2+}.

T HE calcification of the extracellular matrix in growth plate cartilage is the final event of the chondrocyte life cycle. Before this event, the chondrocytes progress through sequential stages of differentiation which are characterized by a high proliferative capacity, extensive matrix production and finally cell hypertrophy (Fell, 1925; Stocum et al., 1979). The molecular events involving the initiation and regulation of cartilage mineralization are not clearly understood. However, it is generally accepted that the macromolecular components of the extracellular matrix surrounding the chondrocytes play an important role in the initiation and progression of calcification (Boskey, 1981; Cowell et al., 1987). Both collagen and noncollagenous matrix macromolecules have been implicated in this process.

Since type X collagen is synthesized by growth plate hypertrophic chondrocytes destined for mineralization (Schmid and Conrad, 1982*a,b*; Kielty et al., 1984, 1985; Kwan et al., 1986), it is thought that this collagen may have a key role in the mineralization of the extracellular matrix. Immunofluorescence analyses (Schmid and Linsenmayer, 1985) have shown that type X collagen is synthesized by chondrocytes before hydroxyapatite accumulation is evident, and it persists in regions of calcified cartilage (Grant et al., 1985; Kwan et al., 1986; Gibson et al., 1986; Lovell and Eyre, 1988; Schmid, et al., 1990) and in spicules of endochondral bone (Schmid and Linsenmayer, 1985).

Although numerous studies reinforce the importance of type X collagen in the calcification process, the exact function of the molecule in this process remains equivocal. Type X collagen has been shown to be associated with alkaline phosphatase (Wu et al., 1991) and matrix vesicles (Habuchi et al., 1985; Wu et al., 1989, 1991), which are considered by some investigators to be the primary nucleation sites involved in cartilage calcification (Bonucci, 1967; Anderson, 1969; Wuthier, 1982).

On the other hand, Poole and Pidoux (1989) could not detect an association of type X collagen with matrix vesicles by immunogold labeling. They suggested type X collagen may prevent calcification of type II collagen and thereby help to focus mineralization in the interfibrillar areas. Arias et al. (1991) have also proposed that type X collagen, or an associated molecule, inhibits calcification based on its presence in the uncalcified inner membranes of the chick eggshell.

Recent studies involving vitamin D–deficient rachitic chick hypertrophic cartilage have suggested that serum calcium levels may affect the synthesis of type X collagen (Reginato et al., 1988; Kwan et al., 1989). Under such vitamin D–deficient conditions, Reginato et al. (1988) reported a relative increase in the concentration of type X collagen compared to collagen types II, IX, and XI. In contrast, Kwan et al. (1989) demonstrated a reduction in mRNA, protein, as well as reduced immunoreactivity for type X collagen in rachitic avian growth plate cartilage. However, when the

vitamin D–deficient diet was supplemented with calcium carbonate, serum calcium levels were normalized and there was a partial recovery of type X collagen synthesis.

The following experiments were initiated to determine the effect of exogenous Ca^{2+} on type X collagen synthesis by chondrocyte cultures.

Materials and Methods

Reagents

Tissue culture reagents were purchased from Gibco Laboratories (Grand Island, NY), except for the defined iron-supplemented calf serum which was from HyClone Laboratories (No. A-2151-L; Logan, UT). L-[2,3,4,5-^{3}H]proline (100 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL).

Chondrocyte Cell Culture

Chondrocytes were isolated from zones 2 and 3 of the hypertrophic cartilage and the tarsus region of the distal end of the tibiotarsus of 12-d chick embryos as previously described (Kim and Conrad, 1977, 1980). The isolated cells from zones 2 and 3 were initially plated in complete Dulbecco's modified Eagle's medium (DMEM), containing 2 g/liter D-glucose, penicillin/streptomycin (100 U/ml), 20% bovine calf serum, and 0.1 mg/ml testicular hyaluronidase (type I). Hyaluronidase was added to promote cell attachment to the culture dishes (Pacifici et al., 1977; Leboy et al., 1989) and had no effect on cell isolations. After 18 h, the attached cells were rinsed two times with DMEM to remove the hyaluronidase and cultured in DMEM plus 10% calf serum for 5 d. Cells prepared from the tarsus region were cultured in the same fashion. However, since tarsal cells demonstrated a high plating efficiency, hyaluronidase was eliminated from the initial cell plating. After 5 d, cells were released with 0.05% trypsin and replated at a density of 2.5 × 10⁴ cells/cm² (for zone 2 and 3 chondrocyte cultures) and 9 × 10⁴ cells/cm² (for tarsal chondrocyte cultures) in complete DMEM containing 10% serum. Cells were allowed to attach to the plastic dishes for 48 h without the addition of hyaluronidase before fresh medium containing 50 μg/ml ascorbic acid and $CaCl_2$ were added. Control cultures received no exogenous Ca^{2+} besides the 1.8 mM Ca^{2+} already found in DMEM, whereas the $CaCl_2$-treated cultures received an additional 5 or 10 mM $CaCl_2$ above this 1.8 mM concentration. Cells received fresh medium every 24 h and were cultured in the absence or presence of additional $CaCl_2$ for a total of 72 h (zone 2 and 3 chondrocytes) or 96 h (tarsal chondrocytes). All cultures were incubated at 37°C in an atmosphere of 7% CO_2 and 93% air.

Isolation of Newly Synthesized Collagen

Cultures were labeled with 25 μCi/ml L-[2,3,4,5-^{3}H]proline in complete DMEM containing 100 μg/ml β-aminopropionitrile fumarate, 50 μg/ml ascorbic acid, and 1% serum. After 24 h, the culture medium was removed and the remaining cell layer was washed with Tris/saline (25 mM Tris, 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 2.5 mM $CaCl_2$, and 0.9 mM $MgCl_2$, pH 7.4). The medium and wash, referred to as the culture medium fraction (CM),[1] were combined and protease inhibitors (1 mM PMSF, 5 mM EDTA, and 10 mM N-ethylmaleimide) were added. The cell layer was extracted overnight at 4°C on an orbital shaker with 135 mM K_2HPO_4, 15 mM KH_2PO_4 (Schmid and Conrad, 1982b), containing 0.1 M EDTA, pH 7.6. The phosphate-EDTA extract was removed and the cell layer washed with cold Tris/saline and combined with the wash. Protease inhibitors were added to the cell extract. This extract is referred to as the matrix fraction (Ma). Both fractions were dialyzed against 0.4 M NaCl, 50 mM Tris, pH 7.5, with 5 mM EDTA, to remove unincorporated radioisotope. To determine total proline incorporation into nondialyzable CM and Ma proteins, an aliquot from both the CM and Ma fractions was counted to determine total extracellular proline incorporation. Our previous studies have shown that the majority of the radiolabeled collagen (>90%), measured as nondialyzable [^{3}H]hydroxyproline was found in these two extracellular pools (CM and Ma) after a 24-h labeling with [^{3}H]proline (Schmid and Conrad, 1982a).

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1. *Abbreviations used in this paper*: CM, culture medium; Ma, matrix fraction.

Gel Electrophoresis and Fluorography

Radiolabeled CM and Ma proteins were separated on 8% SDS–polyacrylamide gels (Laemmli, 1970), with 20,000 cpm applied per lane. At the same time, a standard gel was prepared by loading known quantities per lane (i.e., 2,000–16,000 cpm) of the radiolabeled 59-kD chain of type X collagen, followed by separation on 8% SDS-PAGE. Fluorography was carried out according to Bonner and Laskey (1974) and Laskey and Mills (1975). To quantitate type X and type II collagen in each radiolabeled fraction, dried gels of CM and Ma proteins, as well as a standard gel containing known cpms of type X collagen, were exposed to the same x-ray film and fluorographs were scanned with a laser densitometer (LKB 2202 Ultroscan; Pharmacia-LKB Biotechnology, Piscataway, NJ). A microcomputer and GS365 densitometer software (Hoefer Scientific Instruments, San Francisco, CA) were used to integrate the densitometer signals. Integrated values for each radiolabeled band were recorded as the area under each peak. Graphs of known cpm of type X standards vs. integrated areas were linear when the fluorogram bands had an absorbance (540 nm) between 0.15 and 0.80. Exposure time of both standard as well as CM and Ma fluorograms was adjusted to fall within this linear range for quantitation, and the standard curves were used to determine the cpm of collagen types II and X in each lane. The fraction of each collagen type in both extracellular pools (i.e., cpm collagen band/20,000 cpm) was multiplied by the nondialyzable [^{3}H]proline incorporated into each respective pool to give the total radiolabeled collagen type in each pool. The values for the total radiolabeled collagen types for each culture dish are expressed as the sum of both CM and Ma pools per μg DNA.

Determination of DNA Content

Cell layers were rinsed two times with sterile phosphate buffered saline and digested with 0.5 mg/ml bacterial collagenase (type II; Worthington Biomedical Corp., Freehold, NJ) at 37°C for 18 h in 0.2 M Tris-HCl, pH 7.4. Collagenase was inactivated by the addition of 5 mM EDTA. Dithiothreitol (1 mM) and papain (0.5 mg/ml) were added and the digestion continued for an additional 6 h at 60°C. Culture digests were centrifuged for 5 min and the supernatant was used for the fluorometric assay of DNA (Labarca and Paigen, 1980; Downs and Wilfinger, 1983).

Identification of Type X Collagen by Antibody-Sepharose Precipitation

Radiolabeled chondrocyte proteins were incubated with anti–type X collagen-Sepharose beads that were prepared by coupling a monoclonal antibody (X-AC9) to Sepharose CL-4B with CNBr (March et al., 1974). The antibody beads were incubated with radiolabeled proteins for 18 h at 4°C in 1 M NaCl, 50 mM Tris, pH 7.5, containing 1 mg/ml CHAPS. The beads were rinsed four times with buffer and one time with H_2O and then suspended in Laemmli sample buffer. The samples were heated to 100°C and loaded on SDS-polyacrylamide gels (Solursh et al., 1986).

Results

Culture and Cell Morphology

Mature chondrocytes derived from zones 2 and 3 and less mature chondrocytes derived from the tarsus were isolated from 12-d-old embryonic chick tibiotarsi. The tarsus gave a mixture of small and medium-sized chondrocytes (Fig. 1 A), where cultures from zones 2 and 3 gave larger cells of more uniform size (Fig. 1 B). Some of the cells from the tarsus were slightly elongated, while the cells from zones 2 and 3 showed a polygonal morphology more typical of chondrocytes. During the short incubation period, $CaCl_2$ had no effect on cell morphology in either population of chondrocytes.

Growth Profile of Chondrocyte Cultures

After 48 h in secondary culture, cells were treated with an additional 5 or 10 mM $CaCl_2$ for 3 (zone 2 and 3 chondrocytes) or 4 d (tarsal chondrocytes). Since tarsal chondrocytes
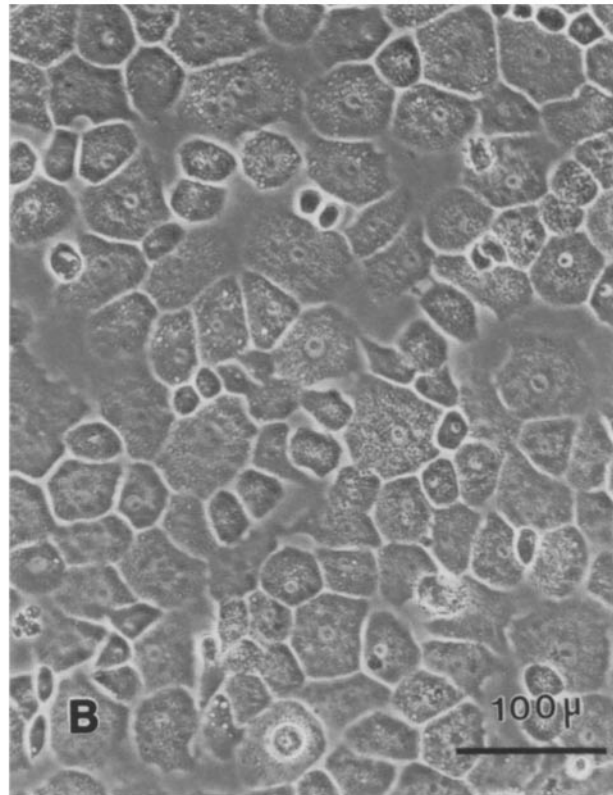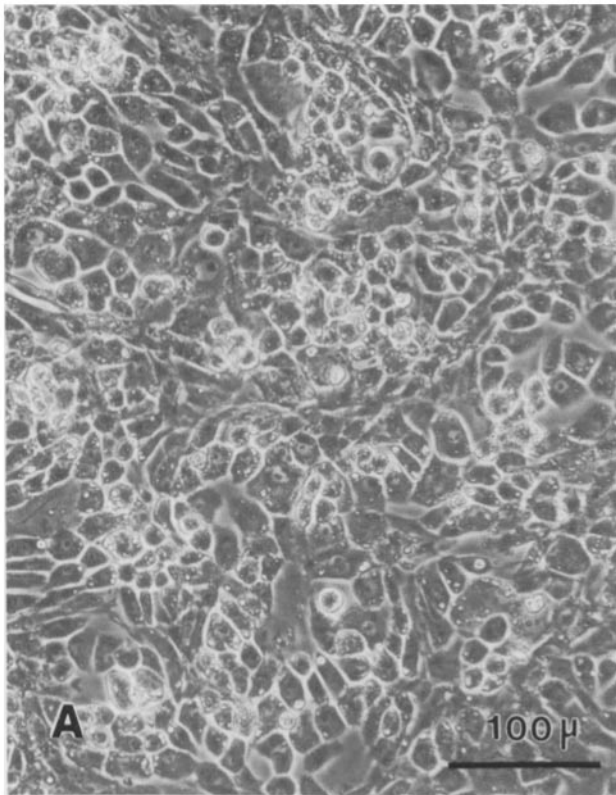
Figure 1. Phase-contrast photomicrographs of primary cultures of chondrocytes isolated from the tarsal element (*A*) and zones 2 and 3 (*B*) of 12-d-old chick embryo tibiotarsi.

were much smaller than zone 2 and 3 chondrocytes, the tarsal chondrocytes were plated at a higher density to ensure confluence at the onset of the experiment. Even though the cell densities were high, the immature tarsal chondrocytes retained their capacity to divide during the experimental period, while zone 2 and 3 chondrocytes did not. The addition of $CaCl_2$ to either chondrocyte population had no effect on cell number (DNA content) during the entire incubation period (data not shown).

Biosynthetic Studies: Radiolabeling of Zone 2 and 3 Chondrocytes

Cultures were labeled at 24-h intervals with [^{3}H]proline over a 3-d period. The amount of incorporation into nondialyzable macromolecules in the medium (CM) and matrix (Ma) increased in a dose-dependent manner with the addition of exogenous calcium (Fig. 2). After the first 24 h, [^{3}H]proline
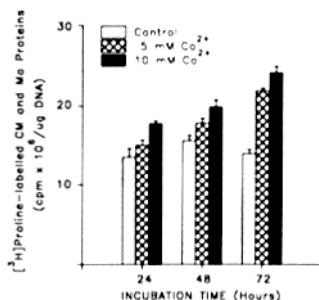

Figure 2. Effect of $CaCl_2$ on the incorporation of [^{3}H]proline into nondialyzable CM and Ma proteins synthesized by zone 2 and 3 chondrocyte cultures. Control cultures are those cells receiving no additional $CaCl_2$. Other cultures were incubated with an additional 5 or 10 mM $CaCl_2$.

incorporation increased 11 and 32% in cultures incubated with 5 and 10 mM $CaCl_2$, respectively, compared with values measured in control cultures. This increase continued at 48 h, with levels of [^{3}H]proline incorporation reaching 14% over control with 5 mM and 27% with 10 mM $CaCl_2$. Cells incubated with calcium for 72 h showed an increase of 56% over controls with 5 mM $CaCl_2$ and 72% with 10 mM $CaCl_2$.

Analysis of Collagen Synthesis by SDS-PAGE: Zone 2 and 3 Chondrocytes

The nature of the [^{3}H]proline-labeled proteins distributed in CM and Ma fractions isolated from zone 2 and 3 chondrocytes were analyzed by SDS-PAGE and fluorography (Figs. 3 and 4). In these fractions, the band with M_r of 59,000 was identified as type X collagen by two criteria: (*a*) comigration with the 59-kD chains of type X collagen (Figs. 3 and 4) and (*b*) precipitation with antibody-Sepharose beads, i.e., X-AC9-Sepharose (Solursh et al., 1986. Fig. 3). The majority of type X collagen was secreted into the culture medium after a 48- (Fig. 3 *A*) and 72-h (Fig. 3 *B*) incubation with calcium. By 72 h, some of the type X collagen also accumulated in the Ma fraction (Fig. 4).

The amount of total extracellular collagen types X and II in nondialyzable CM and Ma fractions was determined by densitometric analysis of CM and Ma fluorograms. Intracellular collagen was not included in the quantitation since only 10% of the collagen, measured as nondialyzable [^{3}H]hydroxyproline, was found in the intracellular pool after a 24-h
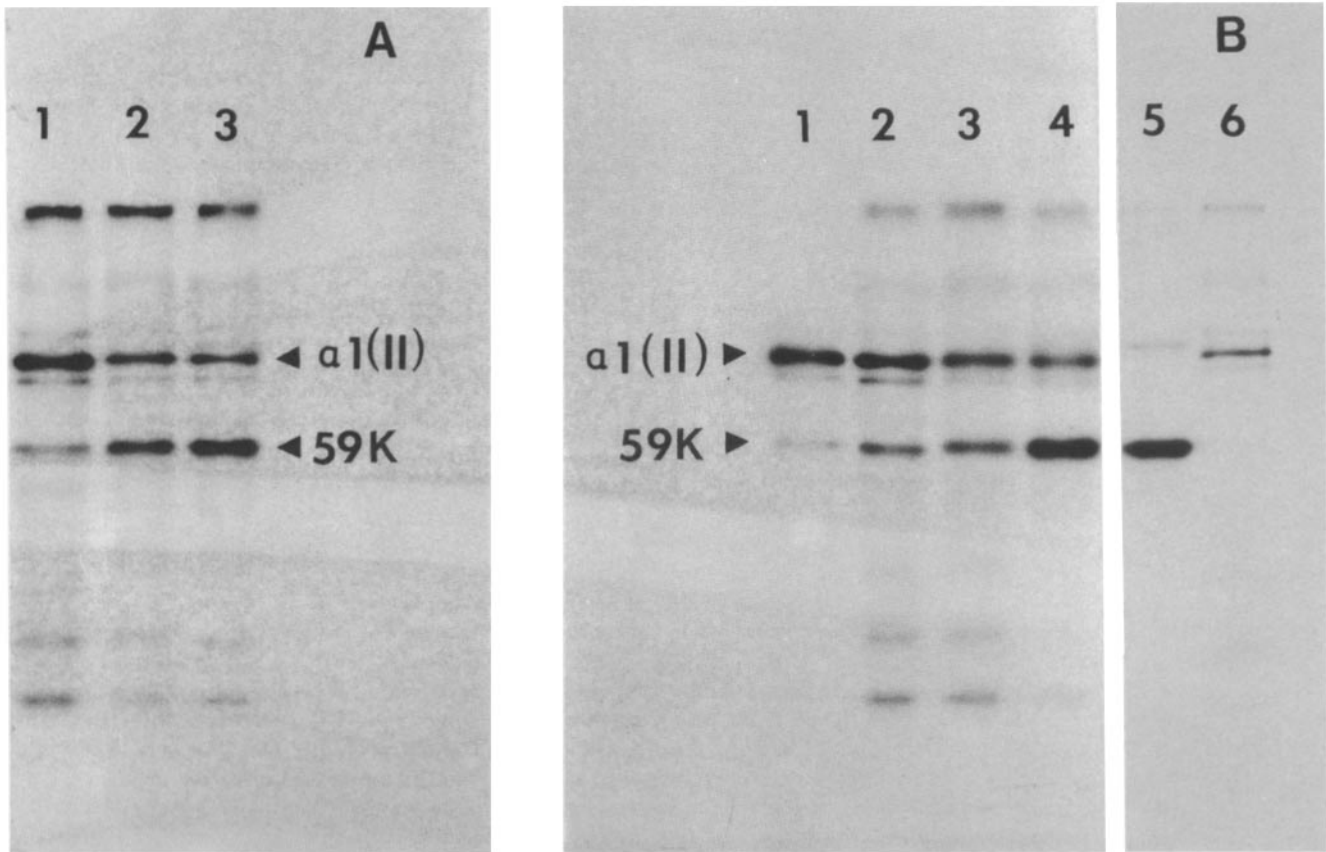
Figure 3. Effect of CaCl₂ on the synthesis of collagen type X secreted into the culture medium by zone 2 and 3 chondrocytes. (*A*) Fluorogram of ³H-labeled culture medium proteins isolated from hypertrophic chondrocyte cultures after a 48-h incubation with additional 5 (lane *2*) and 10 mM CaCl₂ (lane *3*). Lane *1* shows culture medium–labeled proteins from control cultures. Arrowheads mark the position of the α1(II) and 59-kD α1(X) chains. (*B*) Fluorogram of [³H]proline-labeled proteins isolated from culture medium of hypertrophic chondrocytes after a 72-h incubation with 5 (lane *3*) and 10 mM CaCl₂ (lane *4*). Lane *1* contains standards of α1(II) and 59-kD α1(X) chains. Lane *2* shows CM fractions from control cultures. Radiolabeled proteins shown in lane *4* were bound to anti–type X collagen-Sepharose beads (lane *5*). Lane *6* contains radiolabeled proteins from lane *4* not bound to anti–type X collagen-Sepharose beads.

labeling (data not shown), and incubation with CaCl₂ did not change this percentage. Since these chondrocytes incorporated only small amounts of [³H]proline into CM and Ma type I collagen, as evidenced by the low levels of the α2(I) chain, (Figs. 3 and 4), type II collagen was determined by the quantitation of α1(II) chains.

It is apparent that type X collagen synthesis by zone 2 and 3 chondrocytes is markedly influenced by the addition of
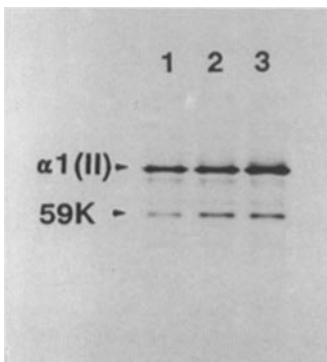

Figure 4. Fluorogram of ³H-labeled proteins extracted from the matrix of zone 2 and 3 chondrocytes grown in the absence (lane *1*) or presence of an additional 5 (lane *2*) or 10 mM CaCl₂ (lane *3*) for 72 h. Each lane contains 20,000 cpm of [³H]proline-labeled proteins.

CaCl₂. In fact, a stimulatory effect on type X collagen synthesis was observed as early as 24 h with type X collagen levels increasing 1.8-fold over controls with the addition of 5 mM and 2-fold with 10 mM CaCl₂ (Fig. 5). At 48 h, this stimulatory response to elevated calcium levels continued with type X collagen levels showing a 2-fold increase over controls with 5 mM CaCl₂ and 2.5-fold with 10 mM CaCl₂. This stimulatory effect of extracellular calcium on type X collagen synthesis was even more evident after 72 h with levels increasing 8-fold over control in 5 mM and 10-fold in 10 mM CaCl₂-treated cultures.

Type X collagen represented up to 10% of the total ³H-labeled CM and Ma proteins synthesized in control cultures after 24 h compared to ~20 and 14% in similar cultures treated with 5 and 10 mM CaCl₂, respectively (Table I). After 48 h in culture, type X collagen accounted for 13% in controls compared to 21 and 24%, respectively, in the 5 and 10 mM CaCl₂-treated cultures. After 72 h in culture, type X collagen represented 9%, whereas in the presence of 5 and 10 mM CaCl₂, the corresponding values were 44 and 51%, respectively.

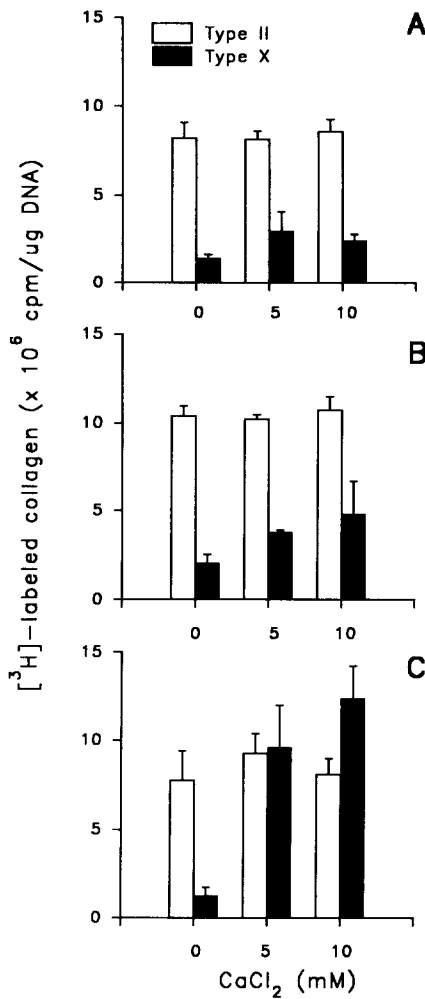During the 72-h incubation, CaCl₂ did not appear to affect the levels of total CM plus Ma type II collagen, even

Figure 5. The influence of Ca^{2+} concentration on the proportion of collagen types X and II synthesized by zone 2 and 3 chondrocytes after (A) 24, (B) 48, and (C) 72 h in culture in increasing concentrations of CaCl$_2$. ^{3}H-labeled collagen levels are the sum of CM and Ma fractions. Values are expressed as the mean ± SE of three separated densitometric measurements.

though type X levels were increased at every time point (Fig. 5). Since type II collagen levels remained relatively constant throughout this 72-h period, it would suggest that the stimulatory effect of extracellular Ca^{2+} is specific for type X collagen.

Induction of Type X Collagen Synthesis in Tarsal Chondrocytes

To determine whether the addition of Ca^{2+} could induce type X collagen synthesis in less mature chondrocytes (i.e., non-hypertrophic chondrocytes), cell cultures were established from the tarsal element of 12-d chick embryo tibiotarsus. These cells differ from hypertrophic chondrocytes in their cell morphologies, their adhesion to each other, their growth rates (Kim and Conrad, 1977) and in the production of type X collagen. The electrophoretic patterns of ^{3}H-labeled CM proteins are shown in Fig. 6. Low levels of type X collagen are detectable in the CM fractions in both control and CaCl$_2$-treated cultures during the first 48 h, with a

Table I. Influence of CaCl$_2$ on the Percentage of Type X Collagen Synthesized by Hypertrophic Chondrocytes Derived from Zones 2 and 3 of 12-d-old Chick Embryo Tibiotarsus

Additional [Ca^{2+}]	Percent type X collagen*		
	24 h	48 h	72 h
mM	%		
0	10.5 ± 1.6	13.0 ± 3.0	9.0 ± 3.3
5	19.8 ± 7.3	21.1 ± 0.6	43.9 ± 11.0
10	13.7 ± 1.8	24.2 ± 9.4	51.3 ± 7.7

* Values for type X collagen represent the percent of total [^{3}H]proline incorporated into CM and Ma proteins and are given as the mean ± SE obtained from two separate experiments.
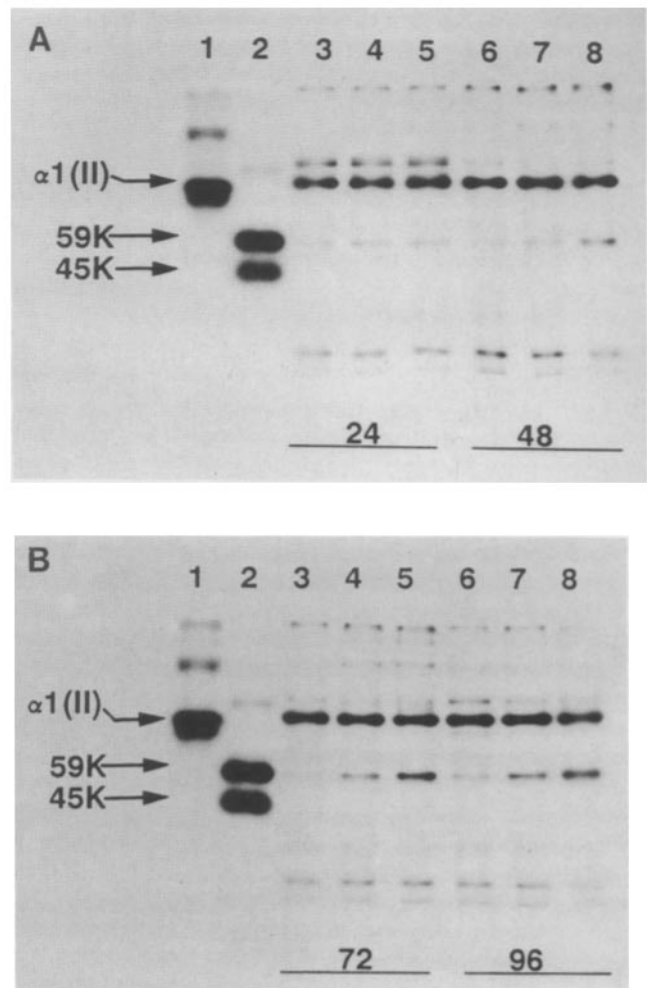

Figure 6. Effect of Ca^{2+} on the synthesis of type X collagen secreted by tarsal chondrocyte cultures. (A) Fluorogram of ^{3}H-labeled culture medium proteins incubated with CaCl$_2$ for 24 (lanes 3-5) and 48 h (lanes 6-8). (B) Fluorogram of ^{3}H-labeled culture medium proteins incubated with CaCl$_2$ for 72 (lanes 3-5) and 96 h (lanes 6-8). In both A and B, cells were cultured with 5 (lanes 4 and 7) or 10 mM CaCl$_2$ (lanes 5 and 8). CM-labeled proteins from control cultures are shown in lanes 3 and 6. Lane 1 shows the position of the α1(II) chain of type II collagen; lane 2 shows the position of the 59,000- and 45,000-M_r chains of type X collagen.
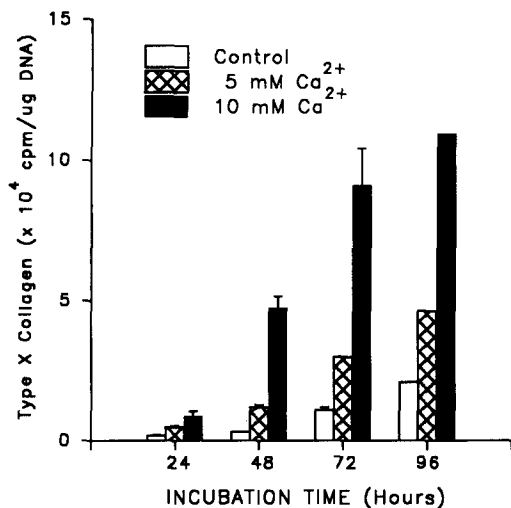
Figure 7. Time course of Ca^{2+} induction of type X collagen in the CM plus Ma fraction of short-term chondrocyte cultures established from the tarsal region of 12-d chick embryos. Values given are based on densitometric analysis of ^{3}H-labeled fluorograms and are expressed as the mean ± SE of two separate experiments.

Table II. Effect of $[Ca^{2+}]$ on the Percentage of $[^3H]Proline$ Incorporation into Type X Collagen Synthesized in Tarsal Chondrocyte Cultures

Additional [Ca²⁺]	Percent type X collagen*			
	24 h	48 h	72 h	96 h
mM		%		
0	0.10 ± .01	0.16 ± .01	0.85 ± .05	1.52‡
5	0.29 ± .03	0.56 ± .04	2.00 ± .01	3.10
10	0.51 ± .10	2.24 ± .20	5.63 ± .83	7.34

* Values for type X collagen represent the percent of total [³H]proline incorporated into CM and Ma proteins and are given as the mean ± SE obtained from duplicate cultures.
‡ Values without ± SE represent experiments that were not done in duplicate.

noticeable increase in type X collagen in the presence of an additional 5 and 10 mM Ca^{2+} after a 48-h incubation (Fig. 6 *A*). This influence of extracellular Ca^{2+} on type X collagen secretion into CM fractions is even more dramatic at both 72 and 96 h (Fig. 6 *B*).

SDS-PAGE analysis of ^{3}H-labeled matrix proteins showed no detectable levels of type X collagen incorporated into the matrix of control or CaCl$_2$-treated cultures after a 24- and 48-h incubation, with a twofold increase in type X collagen observed at both 72 and 96 h with 5 and 10 mM Ca^{2+} (data not shown).

The results obtained from densitometric scanning of the CM and Ma fluorograms are summarized in Fig. 7 and Table II. Percent type X collagen is determined as the proportion of the combined CM and Ma type X collagen, determined by densitometric analysis, in the total nondialyzable CM and Ma radiolabeled proteins. The immaturity of the tarsal chondrocytes is clearly evident by the low levels of type X collagen synthesized by these cells compared to the levels produced by the mature chondrocytes of zones 2 and 3 (compare Figs. 5 and 7). Densitometric analysis of both CM and Ma fluorograms showed that under control conditions, zone 2 and 3 chondrocytes synthesized a total of 1.42 × 10^6 cpm/μg DNA while tarsal cells synthesized 0.18 × 10^4 cpm/μg DNA after the first 24 h of culture. Therefore, zone 2 and 3 chondrocytes produced almost 800 times more type X collagen than the tarsal chondrocytes at this time point. At 96 h, type X collagen produced by tarsal chondrocyte cultures under control conditions accounted for only 1.5% of the total [³H]proline incorporated into newly synthesized CM and Ma proteins (Table II) compared to 9% as seen in the controls of zone 2 and 3 chondrocyte cultures at 72 h (Table I).

It was consistently found that elevated extracellular calcium levels caused a dose- and time-dependent increase in total CM and Ma type X collagen (Fig. 6). The most dramatic induction of type X collagen synthesis by the tarsal

chondrocytes occurred after a 48-h incubation with CaCl$_2$, with type X collagen levels having markedly increased 4-fold over controls with the addition of 5 mM Ca^{2+} and 15-fold with 10 mM Ca^{2+}. This dose-dependent response to extracellular levels of Ca^{2+} continued for the duration of the experiment.

Discussion

The aim of the present study was to investigate the effect of elevated extracellular calcium levels on type X collagen production by chondrocytes exhibiting differences in cellular maturity. Mature chondrocytes derived from zones 2 and 3 of 12-d chick embryo tibiotarsus were cultured in the presence of increasing concentrations of CaCl$_2$ (5 or 10 mM). Under such conditions, newly synthesized total type X collagen (i.e., CM plus Ma) increased in a time- and dose-dependent manner. The most dramatic effect was seen after a 72-h incubation, with type X collagen levels increasing 8-fold over control with an additional 5 mM and 10-fold with 10 mM CaCl$_2$. Since the addition of CaCl$_2$ had no effect on DNA levels during the 72-h period, any increase in type X collagen was not due to cell proliferation.

Measurement of type II collagen revealed no significant effect from CaCl$_2$ during this 72 h in culture, suggesting the synthesis of these two collagens are regulated by different mechanisms.

To determine if there was an actual induction of type X collagen synthesis, similar studies were conducted with less mature chondrocytes isolated from the tarsal element of 12-d chick embryo tibiotarsi. Even though type X collagen levels were lower in the tarsal chondrocyte cultures compared to zone 2 and 3 chondrocyte cultures, there was a marked induction of type X collagen with increased levels of CaCl$_2$. Type X collagen synthesis was elevated as much as fourfold with 5 mM and 15-fold with 10 mM Ca^{2+} as early as 48 h.

The present investigation is the first report that extracellular calcium has a direct effect on the synthesis of type X collagen by chondrocytes in vitro. Previous studies by Grant et al. (1988) and Thomas et al. (1990) have demonstrated that type X collagen synthesis is markedly influenced only when exogenous Ca^{2+} and P$_i$ were added together. Our experiments, however, show that the addition of calcium by itself is sufficient to elicit an induction of type X collagen. These conflicting results could possibly be due to the differences in the culture systems. In any event, the ability of extracellu-

lar calcium to induce, in cultured chondrocytes, changes in type X collagen, a matrix molecule normally associated with chondrocyte hypertrophy, suggests that local calcium levels may modulate the maturation of chondrocytes. In the past, extracellular calcium has been shown to alter the expression of the collagen phenotype in articular cartilage (Deshmuk, 1976) as well as in limb bud chondrogenesis (Bee and Jeffries, 1987). This supports the concept that certain environmental cues play a role in the modulation of cell differentiation and production of differentiation-specific molecules, specifically type X collagen (Solursh et al., 1986; Tacchetti et al., 1987; Bruckner et al., 1989).

To further substantiate the findings presented here that extracellular calcium may play a key role in chondrocyte differentiation and/or the regulation of type X collagen synthesis are the numerous studies involving the calcium-regulating hormone vitamin D. Vitamin D has been implicated as having a crucial role in the maturation of growth plate chondrocytes (Gerstenfeld, 1990a,b). More importantly, vitamin D has recently been shown to modulate the production of type X collagen in vitro. Gerstenfeld et al. (1990a,b) reported a slow response to 1,25(OH)$_2$ treatment, with type X collagen appearing only after 4 d. Therefore, it is a possibility that the increase in type X collagen synthesis was a secondary hormone response to alterations in calcium homeostasis.

The mechanisms by which the chondrocytes respond to changes in extracellular calcium levels have not yet been established. However, extracellular levels of Ca^{2+} have been shown to play a role in cell division and cell activation in various systems (Berridge, 1975; Rasmussen et al., 1972), as well as regulating the distribution and transport of newly synthesized heparan sulfate proteoglycans in parathyroid cells (Takeuchi et al., 1990). It has also been well established that increased extracellular Ca^{2+} levels elicit changes in intracellular calcium concentrations in parathyroid cells (Shoback et al., 1983; Nemeth and Scarpa, 1986; Wallfelt et al., 1988) as well as osteoclasts (Malgorali et al., 1989; Miyauchi et al., 1990) by stimulating Ca^{2+} entry into the cell and the release of Ca^{2+} from intracellular stores. Such fluxes in cytosolic Ca^{2+} concentration have been demonstrated in many cells to play a critical role in the regulation of cellular metabolism (Carofoli et al., 1977; Murphy et al., 1980; Williamson et al., 1985; Williamson and Murphy, 1980). Chondrocytes may respond in a similar manner to changes in extracellular calcium concentrations by altering their intracellular ionized calcium. Therefore, in growth plate chondrocytes, these intracellular changes in calcium may be involved in chondrocyte matrix mineralization.

It has been demonstrated by in situ studies (Martin et al., 1969; Matthews et al., 1970; Brighton and Hunt, 1974, 1978) as well as in individual cell isolation of growth plate chondrocytes (Iannotti et al., 1989; Iannotti and Brighton, 1989) that as the chondrocyte hypertrophies, there is an accumulation of intracellular ionized calcium in the mitochondria. The presence of high concentrations of mitochondrial granules in hypertrophic cartilage and the absence of such granules in rachitic rat growth plate (Matthews et al., 1970) provide further evidence that intracellular calcium may be important in cartilage matrix mineralization. The findings presented here suggest that calcium may be an important signal in the regulation of chondrocyte differentiation.

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